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FULL LENGTH ARTICLE

Environmental isotonicity improves cold tolerance of Nile tilapia, *Oreochromis niloticus*, in Egypt

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Abstract The limited ability of *Oreochromis niloticus* to tolerate low temperatures during winter in temperate and some subtropical region, such as in Egypt, is of major economic concern. The present study was aimed to improve the cold tolerance of Nile tilapia, *O. niloticus*, by using the well documented phenomena of saving energy consumption for osmoregulation in isotonic medium to decrease the physiological response to cold stress at winter months and may solve the Winter Stress Syndrome (WSS) and the over-wintering problems. Fish which were either pre-acclimated to freshwater or isotonic salinity at 25 °C were transferred directly to freshwater or isotonic medium (12‰) at 14 °C. Fish were killed 3, 6, 24, 48, 72 and 168 h after transfer. In the isotonic medium pre-acclimated fish, it is shown that the effect of cold stress on the increment of plasma glucose level was much lower than that in fresh water. From the observations of Na^+, K^+, Mg^{2+}-*ATPase* enzyme activity we conclude that less disturbance of ionic balance caused by cold tolerance was occurred in the isotonic point water than in the fresh water. The results of the acetylcholinesterase specific activity showed that, brain enzyme was inhibited by cold stress, and that the disruption of the cholinergic function induced by cold stress was much more pronounced in fresh water pre-acclimated tilapia than in isotonic point water pre-acclimated fish. Results from this study recommend that pre-acclimation of Nile tilapia, *O. niloticus*, to an environmental salinity close to the isotonicity, before winter onset, may improve their cold tolerance.

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Introduction

The human demand of fish consumption is increased all over the world. Fish is about to become the main alternative source of animal protein. Nile tilapia, *Oreochromis niloticus*, is the predominant cultured species worldwide (Thodesen et al., 2011, 2012; Gjerde et al., 2012; El-Sayed et al., 2012). Tilapia aquaculture represents about 42% of world total aquaculture

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fish production, and about 64% of the total fish production in Egypt (GAFRD, 2011). In Egypt, the tilapia industry is restricted by low temperatures. Enhancement of tolerance to low temperature of Nile tilapia, *O. niloticus*, is important to prolong its growth period, reduce mortality for overwintering and decrease costs.

The optimal temperature for growth of the most tilapia species is between 25 and 28 °C. Reproduction stops at 22 °C and feeding below 20 °C (Costa-Pierce, 2003; El-Sayed, 2006). Tilapia cannot survive temperatures less than 10–12 °C for more than a few days (Ernst et al., 2007). The inability of tilapia to tolerate low temperatures at winter in temperate and some subtropical region, such as in Egypt, is of major economic concern as it causes poor growth and leads to mass mortality during over-wintering (Charo-Karisa et al., 2005).

Osmoregulation is essential to fish life and is energetically expensive. Karsi and Yavuzcan Yildiz (2005), observed that the plasma glucose levels in the Nile Tilapia, *O. niloticus*, exposed to 18‰ salinity for 72 h were relatively high when compared to control in fresh water and the fish exposed to 9‰ salinity. Many studies have revealed that fish in isotonic medium has good physiological conditions. Kang'ombe and Brown (2008), studied the effect of salinity on growth, feed utilization, and survival of Juvenile *Tilapia rendalli* in tanks, the results indicated that salinity of 10‰ is optimal for *T. rendalli* in tank culture. Peterson et al. (2005) determined the cold tolerances of juvenile blue tilapias *O. aureus* at salinities ranging from 0‰ to 35‰ in the laboratory by decreasing temperatures 1 °C/d until fish died, and they found that fish maintained in isosmotic media (11.6‰) survived at lower temperatures than those in water of higher or lower salinity.

It was found that acclimation of teleosts to different environmental salinities causes depletion of energy which is used to stimulate or modulate the operations of various pumps and ion transporters in gill mitochondrion-rich MR cells, which are highly energy-consuming (Chang et al., 2007; Hwang and Lee, 2007; Tseng et al., 2007). Na^+, K^+-ATPase activity increased when tilapia transferred to SW compared with the fish in FW or in isosmotic salinity (Lee et al., 2003).

Acetylcholinesterase [AChE], the lytic enzyme of the cholinergic system, functions in hydrolyzing the neurotransmitter Acetylcholine [ACh] and hence is used as a marker for cholinergic function (Belal and Assem, 2011). The study of Abdel-Halim et al. (2006), stated that activity of cholinesterase in brain and liver of tilapia fish samples collected in winter was lower than in spring and autumn.

The objective of the current study was aimed to improve the cold tolerance of Nile tilapia *O. niloticus*, grown in Egypt by evaluating the changes of plasma glucose, gill ATPase and brain AChE as indicators of a combination of cold and salinity stresses.

Materials and methods

Preparation of the fish for experiments

Fingerlings ranging in total length from 6.0 to 8.0 cm and weighing 8–10 g were used in all experiments. They were obtained from local hatcheries and maintained in 100 L fibreglass tank, at a stocking density of 12 kg fish per tank, under a 12L:12D photoperiod for at least 2 weeks before experiment. The tanks were provided with a continuous flow of aerated dechlorinated tap water at 25 ± 1 °C (hardness 5.2 mg/L as $CaCo_3$; Ca^{2+} 0.045–0.069 mM/L; Na^+ 0.024 mM/L; dissolved oxygen 7.0 ± 0.5 mg/L; pH 7.2–7.5). The fish were fed daily with fish diet (35% protein) at a rate of 3% body weight, feeding was interrupted 24 h prior to the start of experiments and throughout their duration. During the experiments, fish were transferred in groups of 10 fish to 12 L glass aquaria, water was changed every 24 h by siphon technique to minimize disturbance.

Preparation of isotonic medium (IP)

The isotonic point for fish is about 300 ± 5 milliosmol. It is prepared by dissolving 12 g of sea salt in 1000 ml freshwater. The composition of isotonic solution and fresh water are given in the following table.

Medium	Osmolality (mosmol)/kg H_2O	Sodium (mM/L)	Potassium (mM/L)	Calcium (mM/L)	Magnesium (mM/L)
FW	11.6 ± 0.09	1.08 ± 0.03	0.9 ± 0.09	2.9 ± 0.08	0.58 ± 0.62
IP (12)	322.0 ± 1.3	143.6 ± 3.5	3.9 ± 0.03	5.1 ± 0.12	12.9 ± 0.15

Preparation of the cold system

After acclimation of the fish stock in tanks at 25 °C, 10 individuals for each experimental condition were transferred to aquaria set in a controlled cold room. Each aquarium was constantly aerated using an air-stone connected to an air-pump. The temperature of the aquarium water was adjusted to 14 °C. Light was set at 12L:12D photoperiod provided using a lamp controlled by a timer.

Experimental protocol sample collection and analytical techniques

Experimental protocol

Groups of 10 fish were used for each test period for all experiments. Handled controls were subjected to the same amount of disturbance as the experimental fish, including transfer to another aquarium but remained in their original medium. Four experiments were conducted:

1. direct transfer from freshwater (FW) at 25 °C to freshwater (FW) at 14 °C,
2. direct transfer from freshwater (FW) at 25 °C to isotonic point (IP) at 14 °C,

3. direct transfer from isotonic point (IP) 25 °C to freshwater (FW) at 14 °C,
4. direct transfer from isotonic point (IP) 25 °C to isotonic point (IP) at 14 °C.

Sample collection: blood and tissue samples

Groups of 10 fish were killed 3, 6, 24, 48, 72 and 168 h after transfer. Fish were caught by hand net quickly to minimize the disturbance. Then they were placed upside down and the blood was obtained by incision directly into the heart using heparinised glass pipette. Plasma was separated directly by centrifugation trying to avoid haemolysis and stored at −20 °C till analysis. After blood sampling the fish was decapitated, brain was dissected and directly frozen. Individual gill arches and gill filaments were separated and frozen.

Analytical techniques

Glucose concentration was determined in 10 µl plasma using glucose–liquizyme GOD-PAP kit (SPECTRUM, MDSS GmbH Schiffgraben 41 30175 Hannover, Germany, Catalog #250001). The specific activity of acetylcholinesterase (AChE) in brain total homogenate was determined using acetylthiocholine as the substrate and detection with Ellman's reagent (Ellman et al., 1961). Total proteins were determined in each fraction using serum bovine albumin for calibration. The data were expressed as µg equivalents of AChE/mg proteins (Belal and Assem, 2011). Enzyme activity was measured at 37 °C and calculated as the difference between rates of inorganic phosphate liberated in the presence and absence of ouabain (Johnson et al., 1977). The released inorganic phosphate was determined using the method of Fiske and Subbarow (1925). The protein concentration was determined by the method of Bradford (1976) assay. The enzyme activity was expressed as µM Pi/mg protein/h.

Statistical analysis

Data were analyzed using Student's *t*-test. Statistical significance is judged on overlap of 95% confidence intervals ($p < 0.05$). Comparisons were made against controls at 25 °C. The fish transferred from fresh water to fresh water or to isotonic point water were compared with the corresponding values of fresh water acclimated fish as control and those transferred from isotonic point to fresh water or to isotonic point water were compared with the corresponding values of isotonic point acclimated fish as control. Values in the tables are expressed as mean ± standard error of mean (SEM). If there was a significant deviation of the experimental from the control means, the points in the table were marked with one star at level of 0.05 ($p < 0.05$) and two stars if at the level of 0.01 ($p < 0.01$). The program used for statistical analysis is SPSS.

Results

Changes in plasma glucose levels (Table 1)

Glucose levels increased rapidly in plasma of fish transferred from FW (25 °C) to FW (14 °C) to a highly significant level ($p < 0.01$, 72%) compared to control from exposure hour 3 onwards and reached its highest level by exposure hour 48 (77%), thereafter it tended to decrease gradually but remained significantly ($p < 0.01$) higher than control during the rest of exposure time without any sign of recovery. During the rest of experimental conditions glucose in fish plasma increased directly after transfer, thereafter it tend to recovered more or less to control. While recovery occurred by exposure hour 3 when the fish were transferred from FW (25 °C) to IP (14 °C), it takes longer time (168 and 72 h) when the fish were transferred either from IP (25 °C) to FW (14 °C) or from IP (25 °C) to IP (14 °C), respectively.

Changes of gill Na^+, K^+, Mg^{++}-ATPase activities (Table 2)

When the fish were transferred from FW (25 °C) to FW (14 °C) and upon exposure to cold stress, enzyme activity showed gradual decrease till it reached a minimum level by exposure hour 24 ($p < 0.01$, 37.3%) and remained significantly lower than control till the end of experiment. During transfer from FW (25 °C) to IP (14 °C) enzyme activity in gills decreased by cold stress to minimum by exposure hour 24 ($p < 0.01$, 31.6%), thereafter it increased slowly but remained lower than control. After transfer from IP (25 °C) to FW (14 °C) enzyme activity in gills decreased rapidly after transfer ($p < 0.01$, 34.7%) and remained significantly lower than control, by the end of experimental time it showed a slight but significant ($p < 0.05$, 22.6%) increase. The only sign of complete recovery of ATPase activity was recorded when the fish were transferred from IP (25 °C) to IP (14 °C).

Changes of the brain AChE specific activities (Table 3)

During exposure to cold stress after transfer from FW (25 °C) to FW (14 °C) fish brain AChE specific activity decreased

Table 1 Mean values of plasma glucose (mg%) during all experimental conditions.

Time (h)	FW 25 °C to FW 14 °C	FW 25 °C to IP 14 °C	IP 25 °C to FW 14 °C	IP 25 °C to IP 14 °C
0	54.2 ± 2.6	54.2 ± 2.6	58.8 ± 0.0	58.8 ± 0.0
3	93.3 ± 4.9**	83.3 ± 3.0**	135.4 ± 12.9**	69.8 ± 3.5**
6	91.7 ± 8.9**	54.2 ± 2.8	100.0 ± 6.7**	93.5 ± 4.6**
24	91.7 ± 3.7**	56.0 ± 2.4	102.5 ± 2.1**	77.4 ± 3.3**
48	96.4 ± 7.4**	54.2 ± 3.0	105.6 ± 3.2	74.1 ± 6.4**
72	85.8 ± 7.5**	57.7 ± 3.1	104.9 ± 4.9**	58.8 ± 0.0
168	71.9 ± 3.8**	38.9 ± 10.0*	67.6 ± 2.9**	58.8 ± 0.0

* Mean values are significant at the level of $p < 0.05$.
** Mean values are significant at the level $p < 0.01$.

Table 2 Mean values of Na^+, K^+, Mg^{++}-*ATPase* (μM pi/mg pr/h) specific activity in fish gills during all experimental conditions.

Time (h)	FW 25 °C to FW 14 °C	FW 25 °C to IP 14 °C	IP 25 °C to FW 14 °C	IP 25 °C to IP 14 °C
0	136.05 ± 2.31	136.05 ± 2.31	108.29 ± 3.85	108.29 ± 3.85
3	109.97 ± 4.16*	108.54 ± 5.28*	70.71 ± 4.88**	73.79 ± 6.37**
6	110.22 ± 3.88*	96.53 ± 7.19**	63.63 ± 4.90**	68.28 ± 3.49**
24	85.31 ± 3.79**	93.18 ± 8.97**	64.70 ± 5.21**	74.37 ± 8.20**
48	108.31 ± 9.23*	107.35 ± 5.28*	64.42 ± 5.21**	88.13 ± 8.90*
72	109.42 ± 3.62*	107.96 ± 5.80*	75.66 ± 5.72**	108.29 ± 9.27
168	109.51 ± 2.82*	108.20 ± 5.36*	83.84 ± 4.28*	108.28 ± 6.70

* Mean values are significant at the level of $p < 0.05$.
** Mean values are significant at the level of $p < 0.01$.

Table 3 Mean values of brain AChE specific activity (U/mg protein) during all experimental conditions.

Time (h)	FW 25 °C to FW 14 °C	FW 25 °C to IP 14 °C	IP 25 °C to FW 14 °C	IP 25 °C to IP 14 °C
0	0.98 ± 0.182	0.98 ± 0.19	0.84 ± 0.059	0.84 ± 0.06
3	0.97 ± 0.147	0.84 ± 0.24*	0.69 ± 0.060*	0.54 ± 0.12**
6	0.96 ± 0.066	0.79 ± 0.17**	0.54 ± 0.118**	0.53 ± 0.16**
24	0.81 ± 0.126*	0.73 ± 0.12**	0.48 ± 0.070**	0.63 ± 0.08**
48	0.76 ± 0.155*	0.62 ± 0.23**	0.72 ± 0.083*	0.73 ± 0.09*
72	0.70 ± 0.061*	0.95 ± 0.21	0.84 ± 0.142	0.76 ± 0.06*
168	0.70 ± 0.035*	0.91 ± 0.04	0.84 ± 0.156	0.85 ± 0.11

* Mean values are significant at the level of $p < 0.05$.
** Mean values are significant at the level of $p < 0.01$.

gradually till the end of experimental time where the inhibition percentage reached 29% ($p < 0.05$). Upon transferring the fish from FW (25 °C) to IP (14 °C) Brain AChE specific activity decreased by exposure hour 3 ($p < 0.05$, 14%) and it continued to decrease gradually till it reached a minimum by exposure hour 48 ($p < 0.01$, 37%), then recovery occurred from exposure hour 72 onwards. By transferring the fish from IP (25 °C) to FW (14 °C) brain AChE specific activity decreased by exposure hour 3 ($p < 0.05$, 17%) till a minimum was reached at exposure hour 24, ($p < 0.05$, 42%), then it tended to increase till recovery occurred by exposure hour 72. Finally when the fish were transferred from IP (25 °C) to FW (14 °C) brain AChE specific activity decreased by exposure hour 3 ($p < 0.05$, 17%) till a minimum was reached at exposure hour 24, ($p < 0.05$, 42%), then it tended to increase till recovery occurred by exposure hour 72.

Discussion

Winter Stress Syndrome (WSS) is a term coined by Lemly (1993) to describe a condition of metabolic distress in warm-water fish. In the present study and upon cold shock after direct transfer from 25 to 14 °C, fish showed comatose effect and lost equilibrium directly after transfer for about 1 h, this indicates the extreme effect of cold stress on the physical behaviour. This matches with the previous observations done by Hargreaves (2000), who revealed that, tilapias demonstrated comatose behaviour at low temperatures.

In fish plasma glucose levels is one of the most common stress indicators (Assem et al., 2008). In the present experiments plasma glucose levels in fish transferred from FW at 25 °C to FW at 14 °C rapidly increased then, although it tended to decrease slowly, it remained significantly higher than control during the whole experimental period without any sign of recovery, this may indicate a response to cold stress. Hyperglycaemia during cold exposure has been reported in many fish species (Diouf et al., 2000; Kindle and Whitmore, 2006; Giovanna Marino, 2010).

For fish transferred from IP at 25 °C to IP at 14 °C, plasma glucose levels showed slow increase then complete recovery occurred by exposure hour 72. A similar response was found by Karsi and Yavuzcan Yildiz (2005), who observed that plasma glucose levels in the Nile Tilapia, *Oreochromis niloticus*, exposed to 9‰ salinity for 72 h was similar to control in fresh water. These results indicates better response of the fish to cold stress in isotonic media than in fresh water, and more energy may have been directed to overcome the effect of thermal shock.

For fish transferred from FW at 25 °C to IP at 14 °C, the decrease of plasma glucose after 168 h of exposure may be due to a high energy demand so that glucose cannot be accumulated. This may be the result of exposure to two different types of stress, cold stress and salinity change. This resembles the results found by (David et al., 2005).

For fish transferred from IP at 25 °C to FW at 14 °C, also two types of stress (cold stress and salinity change) but the stress was more pronounced than that of the fish transferred from FW at 25 °C to IP at 14 °C, as the plasma glucose peak after 3 h was higher by about 1.5-folds (83.3 and 135.4 mg/dl for experiments 3 and 4, respectively). This indicates that the transfer to the isotonic medium was less stressful than the transfer to fresh water hence represented an additional indicator for the energy saving strategy in the isotonic medium (Assem et al., 1999).

For the isotonic media pre-acclimated fish, it is shown that the effect of cold stress on the increment of plasma glucose level was much lower than that in fresh water adapted fish. Our observations may indicates that in isotonic medium, where there is no osmotic difference between external and internal milieu, and where the least energy requirement for osmoregulation occurs, cold tolerance can be enhanced. In other word and by saving energy for osmoregulation, which represents 20% and 50% of resting metabolic energy (Boef and Payan, 2001), more energy may have been spared that improve better or even optimal cold tolerance. In the present study the changes of plasma glucose levels recorded during adaptation to cold stress may have several reasons: (1) The elevated plasma glucose was necessary for increased metabolic requirements. (2) The changes were the results of water loss without any change in the absolute amount of glucose. The results reported in the present work give clear evidence that mainly the first reason was useful to describe the reaction. In previous study from our laboratory we found that the haematocrit value and plasma water content of fish treated similarly were not changed (Hassan, 2011).

To support further our assumption that isotonicity of the environment may enhance the cold tolerance of *O. niloticus*, we investigated the changes of gill total Na^+, K^+, Mg^{++}-*ATPases* as an important enzyme for the processes of ionic regulation and it is energy dependent as well. Aside from our studies, euryhaline teleosts as *O. niloticus* have to maintain their internal ionic and osmotic balance regardless of fluctuating salinities in the aquatic environment, and that is achieved *via* efficient mechanisms of active salt secretion and absorption in fish gills (Hwang and Lee, 2007; Fiess et al., 2007; Kosztowny et al., 2008).

In our experiments the total enzyme activity in fish acclimated to isotonic point medium was significantly lower than that in fresh water acclimated fish. This indicates that in the isosmotic solution, more energy saving for osmoregulation occurred. This agrees with Lin et al. (2004b), who revealed that after direct transfer of the freshwater (FW) spotted green pufferfish (*Tetraodon nigroviridis*) to fresh water (FW; 0‰), brackish water (BW; 15‰), and seawater (SW; 35‰), the lowest levels of both relative protein abundance and activity of Na^+, K^+-*ATPase* were found in the BW (15‰) group.

Fish exposed to cold stress, either at fresh water or isotonic point, showed a significant decrease in their branchial Na^+, K^+, and Mg^{2+}-*ATPase* enzyme activity. These results are emphasized by those recorded by Sardella et al. (2008) who revealed that after a 2-week acclimation to 15 °C, tilapia experienced osmotic imbalances in both FW and SW. Also agrees with Metz et al. (2003) and Imsland et al. (2003) who found that, there is a positive correlation between temperature and Na^+, K^+-*ATPase* activity. The present recorded inhibition of the enzyme was likely due to low activity of the branchial Na^+, K^+-*ATPase* which has a high temperature isoform (Sardella et al., 2004).

The transfer of fish from FW (25 °C) to FW (14 °C) and from FW (25 °C) to IP (14 °C) caused a significant decrease in gill Na^+, K^+, Mg^{++}-*ATPase* activity with no sign of recovery till the end of the experiment. These observations are in consistent with those from the literature (Sardella et al., 2008) and in good agreement with our assumption.

The response of the isotonic water pre-acclimated fish to cold stress was much better than in fresh water pre-acclimated fish. During transfer from IP (25 °C) to FW (14 °C) the activity of the enzyme began to recovered with gradual increase after 72 h of exposure. And for fish transferred from IP (25 °C) to IP (14 °C) the response to cold stress was much more better as the recovery began at the end of the first day and complete recovery occurred after 72 h of exposure. This indicates that salinity of the medium can minimize the effect of cold stress. Similar to the results of this experiment, Sardella et al. (2008) found that salinity-induced changes in the Na^+, K^+-*ATPase* concentration of mitochondrion rich cells altered the cold tolerance limits of tilapia.

From the observations of Na^+, K^+, Mg^+-*ATPase* enzyme activity we can conclude that the least disturbance of ionic balance caused by cold tolerance is occurred in the isotonic medium. The energy saved from osmoregulation may have contributed to cold tolerance energy requirements.

The changes practiced *in vivo* in the activity of brain AChE may be one of the mechanisms involved in the maintenance of homeostasis by virtue of which fish tended to acclimatize themselves to various forms of stress or environmental complexities. From the results it was observed that during exposure to cold stress, brain AChE specific activity showed a significant decrease, this agreed with Abdel-Halim et al. (2006), who stated that the activity of cholinesterase in brain and liver of tilapia fish samples collected from New Damietta drainage canal in winter was lower than the in spring and autumn. Also Pfeifer et al. (2005), studied the gill AChE activity in relation to temperature and salinity in *Mytilus* sp., and revealed that the AChE activity showed significant seasonal differences with maximum activities during the summer period and minimum activities in winter.

From the results, and upon the direct transfer of the fish from 25 to 14 °C, it was noticed that brain AChE specific activity of fresh water pre-acclimated fish decreased significantly with no sign of recovery till the end of the experiment, while in brain of the isotonic water pre-acclimated fish, the enzyme specific activity, after initial decrease, began to increases and recovery occurred by the end of the exposure time. From these observations it was concluded that, brain AChE was inhibited by cold stress, and that the disruption of the cholinergic function induced by cold stress was more pronounced in fresh water pre-acclimated tilapia than in isotonic pre-acclimated fish.

Conclusion

In conclusion, based on the present research, we find that pre-acclimation of the Nile tilapia *Oreochromis niloticus* to isosmotic salinity in earthen bond is novel, inexpensive and easily disseminated and proves its efficacy in improving the capacity of the fish to withstand the drop of temperature during winter. The isotonicity of the bond could be achieved by the addition of the commercial salt to the bonds before the onset of winter season.

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